Bifidobacterium cuniculi, *Bifidobacterium choerinum*, *Bifidobacterium boum*, and *Bifidobacterium* *pseudocatenulatum*: Four New Species and Their Deoxyribonucleic Acid Homology Relationships

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Among the several thousand bifidobacteria of our collection, 244 strains from the feces of rabbits and suckling pigs, the rumen of cattle, the feces of breast- and bottle-fed infants, the feces of calves, and from sewage are recognized by means of deoxyribonucleic acid (DNA)-DNA hybridization (competition filter method) as belonging to four new, distinct DNA homology groups. Twenty reference DNA preparations from the type strains of the currently known species or from reference strains of the homology groups of *Bifidobacterium* were used for comparison. The phenotypic traits which distinguish these four groups from previously described species of the genus *Bifidobacterium* include gross morphology, fermentation characteristics, guanine plus cytosine content of the DNA, the interpeptide bridge of the cell-wall peptidoglycan, and the transaldolase and 6-phosphogluconate dehydrogenase isozyme pattern (by starch gel electrophoresis). The four groups are named and described as new species of the genus *Bifidobacterium*: *B. cuniculi*, *B. choerinum*, *B. boum*, and *B. pseudocatenulatum*. The type strains of these species are RA93 (=ATCC 27916), SU806 (=ATCC 27686), RU917 (=ATCC 27917), and B1279 (=ATCC 27919), respectively.

Several species and provisional groups of bifidobacteria isolated from the feces of adult rabbits have been named and described (17, 20, 24). One of the provisional groups was referred to by TrovateLLi et al. (24) as deoxyribonucleic acid (DNA) homology group II. In the present study, this group of bifidobacteria, here referred to as the *cuniculi* DNA homology group, is described in detail and is recognized as a new species.

Bifidobacteria from the feces of piglets were isolated and studied by Zani et al. (26). Most of the strains were identified on the basis of DNA homology relationships as members of *Bifidobacterium suis*, *B. thermophilum*, *B. globosum*, or *B. pseudolongum*. These authors also reported that 13 of 95 strains examined were distinct from previously described species. This group was designated homology group *coirinese*. A few fermentation characteristics and the DNA homology relationships to other organisms from this habitat were reported (26), but the taxonomic status of this group was not established. In the present work, this group, referred to here as the *choerinum* DNA homology group, is also described and recognized as a new species.

The rumen is an important habitat of bifidobacteria, especially when the animals are fed carbohydrate-rich diets (25). Indeed, isolates

from sheep rumen were used for our first studies on the fructose 6-phosphate shunt mechanism of glucose degradation by bifidobacteria (15). Species reported from this habitat include *B. globosum* and *B. ruminale*. *B. ruminale* was found to be identical with *B. thermophilum*, a species isolated by Mitsuoka (6) from the feces of swine. Other bifidobacteria from the rumen (strains RU276, RU348, and RU354) at first appeared to be variants of *B. ruminale* (ref. 18, p. 290) with a distinct morphology (ref. 18, Fig. 3 to 5, p. 282); later their DNA relatedness to *B. thermophilum* P16-6 (and *B. ruminale* RU326) was found to be nearly 70% (ref. 19, Table 10, p. 292). In recent investigations on the effect of diet on the bifidobacteria of the calf rumen, additional isolates were studied by DNA-DNA hybridization. Most of the isolates were members of *B. globosum* or *B. thermophilum* (*B. ruminale*); others (RU917, RU928, RU929, and RU930) were distinct from these species and formed homology group IV, which was rather closely related to *B. thermophilum*. DNA homologies between strains of group IV and strains of *B. thermophilum* ranged from 55 to 75% (25). From a survey of 460 strains not previously studied by means of DNA-DNA hybridization, including rumen isolates and strains from the feces of piglets (homology group III of Zani et

TABLE 1. List of isolates studied

Isolates	Source	Culture received from
<i>Bifidobacterium cuniculi</i> sp. nov.		
RA93 (= ATCC 27916) Type strain	Rabbit feces	Our collection
RA94;RA95/ ^a RA98;RA99/RA104;RA105	Rabbit feces	Our collection
<i>Bifidobacterium pseudocatenulatum</i> sp. nov.		
B1279 (= ATCC 27919) Type strain	Infant feces	Our collection
B1280;B1286;B1291;B1297/B1330;B1336;B1337;B1342/B1457;B1458;B1460;B1462	Infant feces	Our collection
B1464;B1466;B1467/B1555;B1557;B1561/B1663;B1666;B1669;B1670/B1735;B1736/	Infant feces	Our collection
B1828;B1830;B1831;B1832;B1833;B1834/B1874;B1875;B1879;B1880/B1886;B1888/	Infant feces	Our collection
B1889;B1892;B1893/B1957;B1960;B1961;B1965;B1966/B1969;B1970;B1973;B1977/	Infant feces	Our collection
B2067/B2072;B2073;B2075;B2077;B2078;B2079;B2080;B2082;B2083/B2247 ^d ;B2248	Infant feces	Our collection
B2249;B2250;B2251;B2252;B2253/B2262;B2263;B2266/B2282;B2283;B2284;B2286	Infant feces	Our collection
B2287;B2288/B2339;B2340/B2351/B2359;B2361;B2362;B2363;B2366;B2367/B2445/	Infant feces	Our collection
B2446;B2451/B2540;B2541;B2542;B2543;B2544;B2545/B2650;B2651;B2652;B2653	Infant feces	Our collection
B2654;B2655;B2656	Infant feces	Our collection
VT6;VT9/VT74;VT75;VT77/VT164;VT167;VT168;VT169;VT170;VT171;VT172	Calf feces	Our collection
VT173/VT174;VT179;VT180;VT183/VT185;VT186;VT187/VT201;VT202;VT203	Calf feces	Our collection
VT204/VT205;VT206;VT207/VT254/VT262;VT263;VT265;VT269;VT270/VT273	Calf feces	Our collection
VT274;VT275;VT276;VT277;VT278/VT279;VT282;VT283;VT284;VT285;VT287/	Calf feces	Our collection
VT337;VT339;VT341/VT348/VT351;VT353;VT354;VT356;VT360/VT368;VT369	Calf feces	Our collection
VT370;VT371;VT372;VT373;VT374;VT375/VT393;VT394;VT395/VT396;VT397	Calf feces	Our collection
VT398;VT399;VT401;VT404/VT405;VT406;VT409	Calf feces	Our collection
<i>Isolates from sewage</i> (starch positive; formerly assigned to <i>B. catenulatum</i> ; see ref. 12, Table 1, p. 7):		
F54/F71/F72/F81/F142/F260;F262/F331/F356;F360/F434/F469	Sewage	Our collection
<i>Bifidobacterium catenulatum</i> Scardovi and Crociani		
B669 (= ATCC 27539) Type strain (12)	Adult feces	Our collection
B660/B661/B676/B684 (= ATCC 27675)	Adult feces	Our collection
B933	Human vagina	Our collection
B1331;B1333;B1334;B1338;B1340;B1341/B1738;B1741;B1742;B1744;B1745;B1746	Infant feces	Our collection
B1748/B1955/B2120;B2121;B2122;B2123;B2124;B2125;B2126;B2127;B2128	Infant feces	Our collection
B2130/B2218/B2238;B2241;B2242;B2243;B2244;B2245/B2316/B2415;B2416;B2417	Infant feces	Our collection
B2418;B2419	Infant feces	Our collection
<i>Isolates from sewage</i> (starch negative; see ref. 12, Table 1, p. 7)		
F94/F216;F217/F257;F261/F276/F286/F320;F324/F364/F457	Sewage	Our collection
<i>Bifidobacterium boum</i> sp. nov.		
RU917 (= ATCC 27917) Type strain	Bovine rumen	Our collection
RU246/RU276;RU277/RU289/RU312;RU313/RU315;RU318/RU331/RU334/RU339	Bovine rumen	Our collection
RU341/RU346;RU347/RU358/RU395/RU397/RU400;RU403/RU447/RU473/RU518/	Bovine rumen	Our collection
RU590/RU608/RU616;RU617/RU627/RU663;RU664;RU665/RU683/RU888/RU928/	Bovine rumen	Our collection
RU929;RU930	Bovine rumen	Our collection
SU865/SU873/SU910;SU911;SU912 (unassigned Homology Group III, see ref.26)	Piglet feces	Our collection
<i>Bifidobacterium thermophilum</i> Mitsuoka (syn. <i>B. ruminale</i> Scardovi et al.)		
P2-91 (= ATCC 25525) Type strain (6); P16-6; 14-44; Nissin	Piglet feces	T. Mitsuoka ^c
RU326 (= ATCC 25866) Type strain of <i>B. ruminale</i> (18)	Bovine rumen	Our collection
RU301/RU303;RU306/RU325/RU333/RU349;RU350;RU351/RU363;RU364;RU365/	Bovine rumen	Our collection
RU366;RU367/RU368/RU398/RU402/RU408/RU409/RU417;RU419/RU424/RU426/	Bovine rumen	Our collection
RU430;RU433/RU439/RU458/RU466/RU470/RU471/RU502/RU516/RU562/RU564;	Bovine rumen	Our collection
RU568;RU569/RU571/RU629/RU647/RU648/RU671;RU673;RU675/RU699/RU718;	Bovine rumen	Our collection
RU725;RU733/RU766/RU780;RU782/RU788;RU790/RU792/RU891;RU892;RU893/	Bovine rumen	Our collection
RU894;RU895/RU897;RU899	Bovine rumen	Our collection
F50/F146/F150/F333;F334/F386/F438;F440;F442;F443;F445/F451;F452/F454	Sewage	Our collection
F456/F460;F466/F489/F491;F493;F494;F499/F523;F524;F534;F536/F539	Sewage	Our collection

^a Shills (/) separate different specimens from which isolates were obtained.

^b The cultures were received in 1970 from G. Reuter, Institut für Lebensmittelhygiene, Freie Universität, Berlin. Since then they have been maintained in our collection.

^c The cultures were received in 1970 from T. Mitsuoka, Institute of Physical and Chemical Research; Yamato-Machi, Saitama, Japan. Since then they have been maintained in our collection.

^d The underlined isolates were used as additional references.

TABLE 1. *continued*

Isolates	Source	Culture received from
<i>Bifidobacterium choerinum</i> sp. nov.		
SU806 (= ATCC 27686) Type strain	Piglet feces	Our collection
SU827/SU836;SU837;SU838;SU840/SU877/SU882;SU885/SU888/SU891/SU936	Piglet feces	Our collection
F390/F441/F447/F527	Sewage	Our collection
<i>Bifidobacterium bifidum</i> (Tissier) Orla-Jensen S28a (= ATCC 15696)	Infant feces	G. Reuter ^b
<i>B. breve</i> Reuter S1 (= ATCC 15700) Type strain (9)	Infant feces	G. Reuter ^b
S50 (= ATCC 15698) (<i>B. parvulorum</i> Reuter)	Infant feces	G. Reuter ^b
<i>B. infantis</i> Reuter S12 (= ATCC 15697) Type strain (9)	Infant feces	G. Reuter ^b
S76e (= ATCC 15702) (<i>B. liberorum</i> Reuter)	Infant feces	G. Reuter ^b
659 (= ATCC 25962) (<i>B. lactentis</i> Reuter)	Infant feces	G. Reuter ^b
<i>B. adolescentis</i> Reuter E298b (= ATCC 15705) Biotype c (8)	Adult feces	G. Reuter ^b
<i>B. longum</i> Reuter E194b (= ATCC 15707) Type strain (9)	Adult feces	G. Reuter ^b
S3 (= ATCC 15708) Biotype b (8)	Adult feces	G. Reuter ^b
<i>B. dentium</i> Scardovi and Crociani B764 (= ATCC 27534) Type strain (12)	Dental caries	Our collection
<i>B. angulatum</i> Scardovi and Crociani B677 (= ATCC 27535) Type strain (12)	Adult feces	Our collection
<i>B. globosum</i> Scardovi et al. RU230 (= ATCC 25864) Type strain (18)	Bovine rumen	Our collection
<i>B. pseudolongum</i> Mitsuoka PNC-2-9G (= ATCC 25526) Type strain (6)	Pig feces	T. Mitsuoka ^c
Mo-2-10 biotype d	Mouse feces	T. Mitsuoka ^c
<i>B. magnum</i> Scardovi and Zani RA3 (= ATCC 27540) Type strain (20)	Rabbit feces	Our collection
<i>B. pullorum</i> Trovatelli et al. P145 (= ATCC 27685) Type strain (24)	Chicken feces	Our collection
<i>B. animalis</i> (Mitsuoka) Scardovi and Trovatelli R101-8 (= ATCC 25527) Type strain (17)	Rat feces	T. Mitsuoka ^c
P23 (= ATCC 27536)	Chicken feces	Our collection
<i>B. suis</i> Matteuzzi et al. SU859 (= ATCC 27533) Type strain (5)	Piglet feces	Our collection
<i>B. asteroides</i> Scardovi and Trovatelli C51 (= ATCC 25910) Type strain (16)	Bee intestine	Our collection
<i>B. indicum</i> Scardovi and Trovatelli C410 (= ATCC 25912) Type strain (16)	Bee intestine	Our collection
<i>B. coryneforme</i> Scardovi and Trovatelli C215 (= ATCC 25911) Type strain (16)	Bee intestine	Our collection
Homology group "minimum" F392 (= ATCC 27538) (17)	Sewage	Our collection
Homology group "subtile" F395 (= ATCC 27537) (17)	Sewage	Our collection

al. [26]), a novel group of bifidobacteria, herein referred to as the *boum* DNA homology group, emerged; it is also described in this study as a new species.

In 1971 Scardovi et al. (19) described homology group *catenulatum* on the basis of 10 strains from human feces and vagina and from sewage. Subsequently, an additional 64 isolates from sewage were added to this group, which was proposed as a new species, *Bifidobacterium catenulatum* (12). Because of the uncertainties in defining the DNA homology values that are valid for separating bacterial species (see ref. 1, 3, and 23) and because the known species of the genus *Bifidobacterium* display interspecific DNA homologies much lower than 40 to 50%, with the exception of *B. pseudolongum* and *B. globosum*, which are 60 to 70% related (21), many strains from sewage have been included in the species *B. catenulatum* even though they are only 65 to 70% related to the type strain, B669 (ref. 12, Table 8, p. 12). Two sets of experimental results indicate that the inclusion of

these strains is probably incorrect. First, as described here, the new rumen homology group is separated from *B. thermophilum* Mitsuoka (*B. ruminale*) on the basis of their intergroup DNA relatedness range (60 to 80%) as determined by a large number of filter-competition tests made with many reference strains from each group. The distinct nature of the peptidoglycan structure and isozyme patterns of these two groups suggests that the DNA homology values do indeed represent distinct entities. Similarly, DNA-DNA hybridization of a large number of isolates from feces of breast-fed infants (more than 1,500 strains) enabled us to recognize the existence of a large group of isolates which, although similar in many respects to *B. catenulatum*, were only 60 to 80% related to B669, the type strain of this species. These strains were also phenotypically distinctive. Identical strains were isolated from the feces of suckling calves. The assignment of all sewage strains to the species *B. catenulatum* therefore has to be reconsidered. The closely related strains from humans and calves are here

TABLE 2. DNA relatedness of *B. cuniculi* and *B. choerinum* to other species of *Bifidobacterium* found in animal feces

Competitor isolates	Percent homology to reference DNA from					
	<i>B. cuniculi</i> RA93	<i>B. choerinum</i> SU806	<i>B. globosum</i> RU230	<i>B. thermophilum</i> RU326	<i>B. pseudolongum</i> Mo-2-10	<i>B. animalis</i> P23
<i>Bifidobacterium cuniculi</i> sp. nov.						
RA93 (= ATCC 27916) Type strain	100	15	51	20	16	25
RA94;RA95/*;RA98;RA99/ RA104;RA105	97-102-95-101 106-94	12-10-9-14 10-10	55-60-50-60 67-52	15-18-12-16 20-20	11-12-8-9 10-8	22-24-20-18 20-18
Range	94-102	9-15	50-67	12-20	8-16	18-25
<i>Bifidobacterium choerinum</i> sp. nov.						
SU806 (= ATCC 27686) Type strain	15	100	48	28	38	18
SU827/SU836;SU837;SU838	10-10-8-10	89-75-75-91	26-44-30-43	34-15-33-29	60-40-42-46	4-5-12-17
SU840/SU877/SU882;SU885/ SU888/SU891/SU936	16-8-9-20 9-8-12	92-80-104-86 76-90-82	57-28-39-36 26-42-30	35-16-24-26 34-30-30	57-48-62-48 49-49-37	20-7-9-13 10-21-11
strains from sewage: F390/F441/F447/F527	*b.*.*	116-113-109-120	*.*.*.*	*.*.*.*	*.*.*.*	*.*.*.*
Range	8-20	75-120	26-57	15-35	37-62	4-20

^a Shills (/) separate different specimens from which isolates were obtained.^b *, Not tested.TABLE 3. DNA homology relationships between the species of the genus *Bifidobacterium* (used as competitors) and the type strains of the new species (used as references)

Competitor strains	Percent homology to reference DNA from			
	<i>B. choerinum</i> SU806 ^a	<i>B. cuniculi</i> RA93 ^a	<i>B. boum</i> RU917 ^a	<i>B. pseudocatenulatum</i> B1279 ^a
<i>Bifidobacterium bifidum</i> S28a	27	10	11	15
<i>B. breve</i> S1; S50	22-20	13-10	18.* ^b	10.*
<i>B. infantis</i> S12; S76c; 659	20-21-22	22-16-23	11.*.*	15.*.*
<i>B. adolescentis</i> E298 b	20	25	25	30
<i>B. longum</i> E194b; S3	17-16	16-19	23.*	22.*
<i>B. catenulatum</i> B669	32	10	8	58
<i>B. dentium</i> B764	15	17	0	18
<i>B. angulatum</i> B677	16	17	10	20
<i>B. globosum</i> RU230	50	45	14	5
<i>B. pseudolongum</i> PNC-2-9G; Mo-2-10	45-50	10-15	10.*	5.*
<i>B. thermophilum</i> P2-91; P16-6; 14-44	16-18-15	15-15-20	70-75-72	*.*.*
Nissin; RU326 (<i>B. ruminale</i>)	20-19	25-17	*-63	*-16
<i>B. magnum</i> RA3	10	10	12	20
<i>B. animalis</i> R101-8; P23	18-20	31-26	*-21	*-10
<i>B. pullorum</i> P145	37	25	13	12
<i>B. suis</i> SU859	21	21	7	25
<i>B. asteroides</i> C51	50	11	0	5
<i>B. indicum</i> C410	44	20	•	•
<i>B. coryneforme</i> C215	36	20	•	•
Homology group "minimum" F392	36	23	0	25
Homology group "subtile" F395	34	14	0	10
<i>B. choerinum</i> SU806	100	16	20	25
<i>B. cuniculi</i> RA93	16	100	5	10
<i>B. boum</i> RU917	23	8	100	5
<i>B. pseudocatenulatum</i> B1279	32	10	5	100

^a Type strains.^b *, Not tested.

regarded as members of a DNA homology group which we refer to as the *pseudocatenulatum* group; the strains of this group are also described below as belonging to a new species.

(A summary of this paper was presented at the Meeting of the Subcommittee on the Taxonomy of Lactobacilli and Bifidobacteria, Amsterdam, 20-23 April 1975.)

MATERIALS AND METHODS

Bacterial strains. The strains included in this study are listed in Table 1.

Determination of phenotypic characters. The methods used to determine the phenotypic characters of the strains studied were previously described (12).

DNA-DNA hybridization. The procedure used for determining DNA relatedness was also performed as previously described (12).

Isozyme patterns. The procedures for starch gel electrophoresis and for staining for transaldolase and 6-phosphogluconate dehydrogenase (6PGD) have been described elsewhere (13). The distribution of the multiple electrophoretic forms of these two enzymes among the known species of *Bifidobacterium* and the numerical identification of these isozymes are reported by Scardovi et al. (11).

TABLE 4. DNA homology relationships between *B. boum* and *B. thermophilum* (*B. ruminale*) determined with five reference strains from each species

Competitor isolates	Percent homology to reference DNA from									
	<i>B. boum</i>					<i>B. thermophilum</i>				
	RU917	RU318	RU608	RU627	RU929	RU326	RU363	RU408	RU647	RU725
<i>B. boum</i> sp. nov.										
RU917 Type strains	100	90	95	88	86	60	56	60	58	68
RU318	102	100	87	84	76	58	52	62	53	72
RU608	81	84	100	93	85	72	58	70	57	76
RU627	85	89	95	100	77	52	56	70	62	73
RU929	92	92	86	90	100	60	49	53	66	70
Range			76 - 102					49 - 76		
<i>B. thermophilum</i>										
RU326 (<i>B. ruminale</i>)	62	60	64	65	50	100	90	94	98	90
RU363	65	71	65	72	60	102	100	95	91	96
RU408	72	41	68	68	60	89	85	100	92	93
RU647	75	72	77	75	43	103	87	90	100	110
RU725	76	55	64	63	41	107	83	89	95	100
Range			41 - 77					83 - 110		
Other competitors:										
<i>B. boum</i>										
RU312	96	88	90	94	n.t. ^a	56	68	57	55	74
RU331	89	87	90	94	n.t.	62	68	63	69	71
RU473	91	96	89	106	n.t.	52	73	68	66	72
RU518	88	91	93	96	n.t.	52	67	57	70	71
RU617	102	88	91	95	n.t.	67	69	62	62	75
Range			88 - 106					52 - 75		
<i>B. thermophilum</i>										
RU368	50	n.t.	77	80	n.t.	99	88	95	n.t.	98
RU433	45	n.t.	65	71	n.t.	83	92	95	93	95
RU629	67	n.t.	75	73	n.t.	100	99	90	99	106
RU673	61	n.t.	66	70	n.t.	108	93	84	101	102
RU699	71	59	62	72	n.t.	106	100	88	91	96
RU766	n.t.	77	70	69	n.t.	n.t.	93	91	n.t.	103
RU780	n.t.	77	70	82	n.t.	n.t.	100	92	n.t.	108
RU782	n.t.	77	77	77	n.t.	n.t.	100	86	n.t.	105
RU788	n.t.	67	69	67	n.t.	n.t.	98	89	n.t.	110
RU790	n.t.	63	67	75	n.t.	n.t.	92	90	n.t.	109
RU792	n.t.	69	66	69	n.t.	n.t.	100	97	n.t.	107
Range			45 - 82					83 - 110		

^a n.t., Not tested.

RESULTS

DNA homology relationships. (i) *cuniculi* DNA homology group. The data in Table 2 show the strong DNA homogeneity of this group of strains compared with reference strain RA93 (=ATCC 27916). Except for *B. globosum* RU230 (50 to 67% homologies), there is little DNA homology with the other strains used in the competition experiments. The *cuniculi* strains showed little (8 to 16%) homology with the type strain, Mo-2-10, of *B. pseudolongum*. These results are further evidence that, as suggested previously (19), *B. globosum* Scardovi et al. (18) is a taxon distinct from *B. pseudolongum* Mitsuoka (6), even though *B. globosum* is cited as a synonym of *B. pseudolongum* in the last edition of *Bergey's Manual* (10). The homology values presented in Table 3 demonstrate that the *cuniculi* group is genetically distinct from 23 described species and subspecies of *Bifidobacterium*.

(ii) *choerinum* DNA homology group. The data in Tables 2 and 3 show the degree of DNA homogeneity of the strains assigned to the *choerinum* group and that the DNAs from most other species or groups of *Bifidobacterium* did not significantly compete in the homologous system with strain SU806 as the reference. It is evident, however, that this group is more closely related to *B. globosum* and *B. pseudolongum* than to any other species of *Bifidobacterium*. The nearly 50% homologies with *B. asteroides* and *B. indicum* were unexpected, indeed; such relatedness between bifidobacteria that are so different in morphology, habitat, and other characteristics merits further investigation in relation to its possible significance in the evolution of *Bifidobacterium* species.

(iii) *boum* DNA homology group. Because of the rather close DNA relatedness of the *boum* DNA homology group to *B. thermophilum* (*B. ruminale*), special attention was given to the assessment of their DNA homology relationships. The data in Tables 4 and 5 show the DNA homology relationships between the strains of this group and *B. thermophilum*. Five reference DNAs and competitor DNAs from additional strains of both groups were used in the reciprocal annealing tests. No overlapping occurred between the ranges of the reciprocal homologies of the two reference groups and those obtained with selected competitors (Table 4). It appears, however, that not all of the reference DNA preparations were suitable for clear-cut discrimination between the two groups of strains. *B. thermophilum* RU725, for example, uniformly showed higher relatedness than did the other reference strains when *boum* group DNAs were

used as competitors (68 to 76%); conversely, reference strain RU929 of the *boum* group gave somewhat lower values when the relatedness of other *boum* DNAs was measured. It follows that if one used only these two references to separate the two groups of strains, the separation would not be very clear. The homology values obtained with reference strains RU326 (*B. thermophilum*) and RU917 (*boum* group) and competitor DNAs from many strains of both groups isolated from rumen, sewage, and feces of piglets are given in Table 5. Although some strains (RU447, RU590, RU663, and RU664) assigned to the *boum* group displayed a relatedness of only 69 to 76% to the *boum* reference strain RU917, their homology to the reference strain RU326 of *B. thermophilum* was much lower. As shown in Table 3, except for *B. thermophilum*, there was little or no DNA homology between the *boum* strains and any of the other strains tested.

(iv) *pseudocatenulatum* DNA homology group. The DNA relatedness of 239 isolates to B1279, the reference strain of the *pseudocatenulatum* group, and B669, the type strain of *B. catenulatum*, is reported in Table 6. The studies generally included multiple isolates from each specimen because the data are part of our investigation of the ecological distribution of *Bifidobacterium* species and biotypes (unpublished). In Table 6, several strains isolated from the feces of infants, e.g., B1280 to B1561, displayed a relatedness greater than 80% to *B. catenulatum* reference DNA; nevertheless, these strains were assigned to the *pseudocatenulatum* group because their homologies to this reference were 20 to 25% higher. We included strains B2247 to B2266 in the *pseudocatenulatum* group although their DNA competed poorly in the two reference systems used. These strains were isolated from two fecal specimens from breast-fed infants. The morphology of these strains was unusual (see Fig. 5, no. 7 and 11); however, these strains all fermented starch, as do other *pseudocatenulatum* strains, and they displayed the same transaldolase and 6PGD isozymes (isozyme 4 and isozyme 3, respectively; see Table 9). When reference DNA was prepared from strains B2247, B2249, and B2262, competition tests (results not tabulated) revealed strong intragroup homology (from 90 to 100%) with the isolates from the same specimens. However, the use of DNA competitors from B669, the type strain of *B. catenulatum*, and B1279 of the *pseudocatenulatum* group did not permit the assignment of these three strains to either group because the two DNAs competed to the same extent: 80% to filters B2247 and B2262 and 75% to filter B2249. The existence of strains intermediate between

TABLE 5. DNA homology relationships between *B. boum* and *B. thermophilum*

Competitor isolates	Percent homology to reference DNA from	
	<i>B. boum</i> RU917	<i>B. thermophilum</i> RU326
<i>B. boum</i> sp. nov.		
Strains from rumen		
RU917 Type strain	100	63
RU246/ ^a RU276;RU277/RU289/RU313/RU315/	83-78-79-79-85-96	67-71-67-67-53-55
RU334/RU339;RU341/RU346;RU347/RU358/	75-95-83-91-81-81	56-64-59-69-59-62
RU395/RU397/RU400;RU403/RU447/RU590/	87-84-76-81-76-75	47-55-57-59-46-59
RU616/RU663;RU664;RU665/RU683/RU888/	93-69-72-81-88-80	70-42-36-64-65-69
RU928/RU930/	95-91	67-74
Strains from feces of piglets		
SU910;SU911;SU912/SU865/SU873/	91-88-92-80-96	63-50-45-36-59
Range	69-96	36-74
<i>B. thermophilum</i>		
Strains from rumen		
RU326 (<i>B. ruminale</i>)	63	100
RU301/RU303;RU306/RU325/RU333/RU349	52-53-49-57-66-59	89-85-86-106-100-93
RU350;RU351/RU364;RU365/RU366;RU367/	48-56-48-54-47-50	88-94-110-102-95-85
RU398/RU402/RU409/RU417;RU419/RU424/	41-45-62-56-55-54	83-84-91-92-99-88
RU426/RU430/RU439/RU458/RU466/RU470/	48-55-42-45-47-40	79-100-106-86-85-97
RU471/RU502/RU516/RU562/RU564;RU568	59-80-73-67-57-62	101-100-106-95-83-101
RU569/RU571/RU648/RU671;RU675/RU718	53-65-71-55-67-46	83-110-106-103-117-93
RU733/RU891;RU892;RU893/RU894;RU895/	63-32-71-57-62-52	97-92-92-92-92-84
RU897;RU899/	27-56	97-97
Range	27-80	79-117
Strains from sewage		
F50/F146/F150/F333;F334/F386/	40-42-37-42-48-35	80-82-87-90-74-82
F438;F440;F442;F443;F445/F451	40-48-42-43-39-30	83-94-90-102-84-85
F452/F454;F456/F460;F466/F489/	44-35-15-36-40-26	90-82-83-96-96-90
F491;F493;F494;F499/F523;F524	51-34-34-34-30-51	85-86-88-79-88-70
F534;F536/F539/	35-34-23	83-91-84
Range	23-51	74-102

^a Skills (/) separate different specimens from which isolates were obtained.

the *pseudocatenulatum* group and *B. catenulatum*, which cannot be distinguished by reciprocal homology tests with the references used in this study, might be expected because of the close relatedness of the two. However, the nature of these intermediate strains should be investigated further. Three strains—VT74, VT75, and VT77—isolated from the same specimen of calf feces also behaved differently (see Table 6). Their DNA competed to the same low extent in both reference systems (even when 300 µg of competitor DNA was used per test); however, unlike the cases described above, the use of strain VT74 as a reference established a clear-cut difference between the *B. catenulatum* and

pseudocatenulatum DNA competitors used (Table 7). Their phenotypic characters confirm this separation. The data reported in Table 3 show that, except for *B. catenulatum*, there was little or no homology between the DNA of the *pseudocatenulatum* strains and any other DNA used in the competition experiments.

Distinctive phenotypic characteristics of the DNA homology groups. (i) Morphology. The general morphology and arrangement of the cells of the DNA homology groups are given in Fig. 1-3, 5, and 6. The morphology of the *boum* strains (Fig. 3) is comparable to that of strains of *B. thermophilum* (Fig. 4). Although the morphology of these organisms is not as distinctive

TABLE 6. DNA homology relationships between *B. pseudocatenuatum* and *B. catenuatum*; type strains used as references

Competitor isolates	Percent homology to reference DNA from <i>B. pseudocatenuatum</i> B 1279	<i>B. catenuatum</i> B 669
<i>B. pseudocatenuatum</i> sp. nov.		
<i>From feces of infant</i>		
B1279 (=ATCC 27919) Type strain	100	60
B1280;B1286;B1291;B1297/ * B1330;B1336	108-100-114-97-109-108	88-79-88-75-59- ^{a,b}
B1337;B1342/B1457;B1458;B1460;B1462	115-101-101-106-101-104	79- ^a -80-83-79-82
B1464;B1466;B1467/B1555;B1557;B1561/	110-104-100-97-112-110	81-81-76-46-78-78
B1663;B1666;B1669;B1670;B1735;B1736/	96-100-93-98-91-93	70- ^a -70- ^a -65-62
B1828;B1830;B1831;B1832;B1833;B1834/	78-99-90-90-86-90	59-67-73-69-70-68
B1874;B1875;B1879;B1880;B1886;B1888/	89-94-84-92-90-95	62-76-66- ^a -65-68
B1889;B1892;B1893/B1957;B1960;B1961	88-92-95-87-80-85	64-75-70-72-72-74
B1965;B1966/B1969;B1970;B1973;B1977/	104-94-106-100-95-85	74-74-68-63-65-68
B2067/B2072;B2073;B2075;B2077;B2078	95-92-91-84-96-80	70- ^a -58-52- ^a -69
B2079;B2080;B2082;B2083/B2247;B2248	82-80-80-85-66-61	69-60-59-72-58-66
B2249;B2250;B2251;B2252;B2253/B2262	<u>68-65-58-65-62-58</u>	65-64-55-58-58-55
B2263;B2266/B2282;B2283;B2284;B2286	<u>63-58-101-112-98-104</u>	62-56-82-87-74-78
B2287;B2288/B2339;B2340/B2351;B2359	101-107-106-85-82-93	74-82-68-52-50-58
B2361;B2362;B2363;B2366;B2367/B2445/	99-89-98-94-102-98	46-70-77-74-79- ^a
B2446;B2451/B2540;B2541;B2542;B2543	107-108-104-96-90-85	85-64-61- ^a - ^a -55
B2544;B2545/B2650;B2651;B2652;B2653	110-105-99-89-87-85	75-72-55-52-57-56
B2654;B2655;B2656	100-115-96	63-64-51
Range	78 - 115 ^c	46 - 88
<i>From sewage (starch positive)</i>		
F54/F71/F81/F142/F260	114-106-108-95-95-95	82-67-69-70-70-68
F 262/F331/F356;F360/F434/F469/	98-89-109-113-102-85	60-56-68-83-76-65
Range	85 - 114	60 - 82
<i>From feces of calf</i>		
VT6;VT9/VT74;VT75;VT77/VT164	90-85-60-58-65-115	60-62-55-50-60-85
VT167;VT168;VT169;VT170;VT171;VT172	81-90-88-100-88-86	67-60- ^a -70-65-62
VT173/VT174;VT179;VT180;VT183/VT185	109-90-89-87-89-109	71-69-71-68-68-77
VT186;VT187/VT201;VT202;VT203;VT204/	96-101-88-87-94-105	70-61-58-59-68-71
VT205;VT206;VT207/VT254/VT262;VT263	114-109-104-109-108-90	72-69-73-85-80-75
VT265;VT269;VT270/VT273;VT274;VT275	89-100-96-100-93-81	75-78-80-82-80-69
VT276;VT277;VT278/VT279;VT282;VT283	90-92-92-88-91-94	79-70-79-73-70-73
VT284;VT285;VT287/VT337;VT339;VT341/	93-88-92-84-80-91	79-75-78-50-64-47
VT348/VT351;VT353;VT354;VT356;VT360/	95-85-93-92-79-91	48-64-65-61-47-40
VT368;VT369;VT370;VT371;VT372;VT373	99-96-85-100-115-111	76-65- ^a -75-81-82
VT374;VT375/VT393;VT394;VT395/VT396	95-102-114-115-100-96	73-79-66-68-75-64
VT397;VT398;VT399;VT401;VT404/VT405	100-101-103-101-89-96	67-69-74-60-69-77
VT406;VT409	85-104	70-64
Range	79 - 115 ^c	40 - 85
<i>B. catenuatum</i>		
<i>From feces of adult man</i>		
B669 (=ATCC 27539) Type strain	58	100
B660/B661/B676/B684	52-50-45-52	90-85-102-85
<i>From human vagina B933</i>		
	65	95
<i>From feces of infant</i>		
B1331;B1333;B1334;B1338;B1340;B1341/	70-60-67-80-75-71	92-80-88-98-101-100
B1738;B1741;B1742;B1744;B1745;B1746	60-76-62-50-56-59	83-93-96-80-83-85
B1748/B1955;B2120;B2121;B2122;B2123	59-55-56-56-60-67	84-82-80-85-90-81
B2124;B2125;B2126;B2127;B2128;B2130/	55-58-60-60-54-62	86-82-92-88-82-90
B2218/B2238;B2241;B2242;B2243;B2244	44-63-56-63-60-51	80-80-81-85-82-79
B2245/B2316/B2415;B2416;B2417;B2418	59-60-60-75-74-76	78-81-91-90-98-100
B2419	71	95
Range	44 - 80	78 - 101
<i>From sewage (starch negative)</i>		
F94/F216;F217/F257;F261/F276/	60-50-55-50-60-54	86-78-85-80-85-93
F286/F320;F324/F364/F457/	54-59-56-58-70	84-87-82-80-86
Range	50 - 70	80 - 93

^a Shills (/) separate different specimens from which isolates were obtained.

^b *, Not tested.

^c Underlined figures were not considered.

TABLE 7. DNA relatedness of some strains from calf feces to *B. pseudocatenulatum* and *B. catenulatum*

Competitor isolates	Percent homology to reference DNA from		
	Strain VT74 (from calf)	<i>B. pseudocatenulatum</i> B1279	<i>B. catenulatum</i> B669
<i>B. pseudocatenulatum</i>			
B1279 Type strain	91	100 ^a	60 ^a
B1280;B1330;B1555;B1663	99-93-94-96	108-109-97-96	88-59-46-70
B1669;B1736;B1830;B1879	107-106-109-95	93-93-99-84	70-62-67-66
B1892;B2073	100-94	92-91	75-58
Range	91-109	91-109	58-88
<i>B. catenulatum</i>			
B669 Type strain	73	58	100
B1331;B1340;B1738;B1741	50-62-75-64	70-75-60-76	92-101-83-93
B1748;B2130	50-74	59-62	84-90
Range	50-75	58-76	84-101
<i>Strains from feces of calf:</i>			
VT74 reference strain	100	60	55
VT75;VT77	94-105	58-65	50-60

^a The figures of these two columns are taken from Table 6.

as the morphology of others, such as *B. bifidum*, *B. angulatum*, *B. breve*, *B. asteroides*, *B. pul-lorum*, etc., distinguishing features can be recognized, especially when the cells are grown on solid TPY medium (Trypticase-phytone-yeast extract-glucose medium; BBL Microbiology Systems). These features are included in the detailed descriptions of these organisms (see below).

(ii) **Fermentation patterns.** The fermentation patterns of the *cuniculi*, *boum*, and *choer-inum* groups are reported in Table 8. The lack of raffinose fermentation is characteristic of *cun-iculi* strains. This negative characteristic is also common to *B. bifidum* and to the "minimum" homology group (17). The carbohydrates fermented by *boum* strains are the same as those fermented by *B. thermophilum* (*B. ruminale*) (6, 18). *choerinum* strains do not possess a fermentation pattern that differentiates them from other organisms. The only difference between the *pseudocatenulatum* and the *B. catenulatum* strains was in the fermentation of starch (and dextrin): all of the *pseudocatenulatum* strains fermented starch, whereas none of the *B. catenulatum* strains did. Strain B669 (=ATCC 27539), the type strain of *B. catenulatum*, has erroneously been reported as fermenting starch (see ref. 12, Table 11, p. 17).

(iii) **Amino acid composition of the cell wall peptidoglycan.** The interpeptide bridges of the cell wall mureins of the reference strains of the four homology groups are as follows (Kandler, personal communication): *cuniculi*, Orn(Lys)-Ala(Ser)-Ala₂; *choerinum*, Orn(Lys)-

Ser-Ala₂; *boum*, Lys-Ser-Glu; and *pseudocatenulatum*, Lys-Ala₂-Ser. Kandler and Lauer (4) reported that the structure of the interpeptide bridge in *B. thermophilum* was of the type Orn(Lys)-Glu, which is distinct from that of the *boum* homology group. The Lys-Ser-Glu type found in the *boum* group is a type previously unknown among bifidobacteria. According to these authors (4), the cell wall peptidoglycan of the type strain of *B. catenulatum* (strain B669) was of the Lys-Asp type, whereas the interpeptide bridge in *pseudocatenulatum* strain B1279 is Lys-Ala₂-Ser, a type also found in *B. magnum* (20). The differentiation of the *boum* and *pseudocatenulatum* groups from *B. thermophilum* and the starch-hydrolysis-negative strains of *B. catenulatum*, respectively, appears to be possible on the basis of their type of cell-wall peptidoglycan.

(iv) **G+C content of DNA.** The guanine plus cytosine (G+C) contents of the DNAs of the four groups were: *cuniculi*, 64.1 ± 0.35 mol%; *choerinum*, 66.3 ± 0.15 mol%; *boum*, 60.0 ± 0.2 mol%; and *pseudocatenulatum*, 57.5 ± 0.3 mol%. The value for the *choerinum* group is high for bifidobacteria, but 66 to 68 mol% G+C was reported for *B. pullorum* (24). The value for the *boum* group does not differ from that reported for *B. thermophilum* (10), but the value for the *pseudocatenulatum* group is significantly higher than the value (54.7 ± 0.3 mol%) reported for *B. catenulatum* (12).

(v) **Isozyme patterns of transaldolase and 6PGD.** Recent investigations (11) on more than 1,200 strains representing all the currently

FIG. 1. Morphology of *Bifidobacterium cuniculi* sp. nov. (1) Cells of the type strain, RA93, from the first anaerobic stab culture (TPY medium) after isolation (July 1972). (2 and 3) Cells of strains RA99 and RA105, respectively, as above. (4) Cells of strain RA93, received as ATCC 27916, from the first transfer in TPY medium (anaerobic stab). (5) Cells of strain RA93 from a surface colony in TPY medium. (6) Cells of the type strain from liquid culture in TPY medium with glucose. (7 and 9) Cells of the type strain from starch and maltose broth cultures, respectively. Phase contrast. Bars, 5 μ m. (8) Two-day-old surface colonies of the type strain, RA93, on TPY medium. Bar, 1 mm.

known species or homology groups of *Bifidobacterium*, concerning the presence and distribution of isozymes of transaldolase and 6PGD, have shown that at least 14 and 19 forms of these two enzymes, respectively, can be detected by means of starch gel electrophoresis. Most species or

groups of bifidobacteria displayed a characteristic set of isozymes by virtue of their widely differing migration rates. The zymogram technique applied to these enzymes therefore constitutes an additional tool for the differentiation of the species of *Bifidobacterium*. Data summa-

FIG. 2. Morphology of *Bifidobacterium choerinum* sp. nov. (1) Cells of the type strain, SU806 (=ATCC 27686), from an anaerobic stab culture (TPY medium). (2 to 5) Cells of strains SU840, SU877, SU838, and SU827, respectively, from the first anaerobic stab culture (TPY medium) after isolation (February 1969). (6 and 7) Cells of the type strain from liquid culture (TPY medium). (8 to 10) Cells of strains SU885, SU882, and SU837, respectively, from liquid cultures (DNA extraction). (11) Cells of strain F390 from the first anaerobic stab culture (TPY medium) after isolation (October 1970). (12) Cells of strain F390 from liquid culture (DNA extraction). (13) Cells of strain SU837 from 6-day-old liquid culture (TPY medium with glucose). (14) Cells of strain F447 from liquid culture (DNA extraction). (15) Cells of strain F527 from anaerobic stab culture (TPY medium). Phase contrast. Bars, 5 μ m. (16) Two-day-old colonies of the type strain, SU806 (TPY medium). Bar, 1 mm.

FIG. 3. Morphology of *Bifidobacterium boum* sp. nov. (1) Cells of the type strain, RU917, from an anaerobic stab culture (TPY medium). (2) Cells of the type strain from surface colonies of TPY medium [see (23)]. (3 to 6) Cells of strains RU318, RU929, RU608, and RU279, respectively, from anaerobic stab cultures (TPY medium). (7) Cells of strain RU334 from broth culture (DNA extraction). (8 to 11) Cells of strains RU447, RU312, RU315, and RU358, respectively, from anaerobic stab cultures (TPY medium). (12) Cells of strain RU312 from a slant culture under CO₂-air. (13 to 17) Cells of strains RU289, RU347, RU334, RU627, and RU315, respectively, from anaerobic stab cultures (TPY medium). (18) Cells of strain SU911 from an anaerobic stab culture (TPY medium). (19 to 21) Cells of strains SU910, SU912, and F375, respectively, from broth cultures (DNA extraction). (22) Cells of strain SU873 from an anaerobic stab culture (TPY medium). Phase contrast. Bars, 5 μ m. (23) Two-day-old surface colonies of the type strain, RU917 (=ATCC 27917), on TPY medium. Bar, 1 mm.

FIG. 4. Morphology of *Bifidobacterium thermophilum* (*B. ruminale*). (1 and 2) Cells of strain RU326 (type strain of *B. ruminale*) from broth cultures (TPY medium). (3 and 4) Cells of strains RU326 and RU363, respectively, from anaerobic stab cultures (TPY medium). (5) Cells of strain P2-91 (type strain of *B. thermophilum*, =ATCC 25525) from a broth culture (DNA extraction). (6 to 8) Cells of strains RU408, RU647, and RU725, respectively, from broth cultures (DNA extraction). (9 and 10) Cells of strains RU301 and RU349, respectively, from anaerobic stab cultures (TPY medium). (11) Cells of strain RU346 from a slant culture under CO₂-air. (12 and 13) Cells of strains RU366 and RU402, respectively, from broth culture (TPY medium). (14 and 15) Cells of strains RU430 and RU458, respectively, from anaerobic stab cultures (TPY medium). (16) Cells of strain RU458 from broth culture (TPY medium) (17 to 21) Cells of strains RU471, RU516, RU562, RU788, and RU790, respectively, from anaerobic stab cultures (TPY medium). Phase contrast. Bar, 5 μ m.

FIG. 5. Morphology of *Bifidobacterium pseudocatenulatum* sp. nov. Strains isolated from infant feces. (1) Cells of the type strain, B1279, received as ATCC 27919, from the first transfer in TPY medium (anaerobic stab culture). (2) Cells of the type strain from a surface colony on TPY medium [see (20)]. (3 to 6) Cells of strains B1970, B2287, B2351, and B2072, respectively, from anaerobic stab cultures (TPY medium). (7) Cells of strain B2262 from an anaerobic stab culture (TPY medium). (8 to 10) Cells of strains B2282, B2284, and B2286, respectively, from the first anaerobic stab culture (TPY medium) after isolation (December 1974). (11 to 16) Cells of strains B2249, B1666, B2544, B1977, B2653, and B2654, respectively, from anaerobic stab cultures (TPY medium). (17 to 19) Cells of strains B2282, B2284, and B2286, respectively, from anaerobic stab cultures [April 1976, see (8) to (10)]. Phase contrast. Bar, 5 μ m. (20) Two-day-old colonies of the type strain, B1279 (TPY medium). Bar, 1 mm.

rized in Table 9 show the isozymes (numbered according to their decreasing migration toward the anode) that have been detected in the strains of the four groups under study. The relative electrophoretic migration of these isozymes is illustrated in Fig. 7. The differences between the isozyme sets of *boum* strains and *pseudocatenulatum* strains and those of *B. thermophilum* and *B. catenulatum*, respectively, add phenotypic evidence for the taxonomic separation of these organisms.

Physiological and biochemical characters. With respect to the physiological and biochemical characteristics that were investigated—relationships to oxygen and CO₂, tem-

perature and pH relationships, end products from glucose, and biochemical reactions—the four groups do not differ from most other bifidobacteria.

DISCUSSION

We have used DNA-DNA homology values as the primary basis for differentiating the species of the genus *Bifidobacterium*. Many authors (see Brenner [1]) accept 70% DNA homology as the "species" level of relatedness and 60 to 70% homology as the level of "geno-subspecies" relatedness (3). However, some of the earlier proposed (6, 8) species of *Bifidobacterium* showed

FIG. 6. Morphology of *Bifidobacterium pseudocatenulatum* sp. nov. Strains isolated from the feces of suckling calves. (1) Cells of strain VT164 from a broth culture (DNA extraction). (2 to 8) Cells of strains VT187, VT399, VT279, VT171, VT282, VT164, and VT183, respectively, from anaerobic stab cultures (TPY medium). (9 to 11) Cells of strains VT356, VT337, and VT270, respectively, from anaerobic stab cultures (TPY medium). (12) Cells of strain VT178 from a broth culture (DNA extraction). Phase contrast. Bar, 5 μ m.

TABLE 8. Fermentation patterns of *B. cuniculi*, *B. boum*, and *B. choerinum*^a

Compound	<i>B. cuniculi</i> 7 strains	<i>B. boum</i> 41 strains	<i>B. choerinum</i> 16 strains
Fructose	- ^b	+	- ^b
L-Arabinose	+	-	-
D-Xylose	+	-	-
Lactose	-	- ^c	+
Raffinose	-	+	+
Inulin	-	+	-

^a All strains fermented glucose, galactose (weak fermentation in some *B. boum* strains), sucrose, maltose, melibiose (see below), starch, and dextrin. Melibiose, fermented by all strains of *B. boum* and *B. choerinum*, was not fermented by the type strain of *B. cuniculi* (ATCC 27916) (W. E. C. Moore, personal communication); however, we repeatedly obtained positive results with this strain. None fermented ribose (weak and occasional fermentation in *B. choerinum*), mannose, trehalose, cellobiose, melezitose, mannitol, sorbitol, gluconate, salicin, glycerol, rhamnose, or lactate.

^b The behavior toward fructose seems to be influenced by the reducing conditions of the medium: fermentation is positive in prerduced media (W. E. C. Moore, personal communication).

^c Ten strains of this species fermented lactose: RU276, RU346, RU397, RU400, RU403, SU865, SU873, SU910, SU911, and SU912.

TABLE 9. Transaldolase and 6PGD isozyme distribution in the four new species and in *B. thermophilum* and *B. catenulatum*

Species and sources of strains	Number of strains	Isozyme of	
		Transaldolase	6-PGD
<i>B. cuniculi</i> from rabbit feces	7 ^c	1 ^a (7) ^b	4 ^a (7) ^b
<i>B. choerinum</i> from piglet feces from sewage	13 2	3 (13) 3 (2)	4 (13) 4 (2)
<i>B. boum</i> from rumen	19	6 (19)	8 (10) 9 (6) 9a (3)
from piglet feces	3	6 (3)	9 (3)
<i>B. thermophilum</i> from rumen	39	8 (39)	7 (10) 8 (12) 9 (16) 9a (1)
from sewage	21	8 (21)	8 (5) 9 (16)
from pig feces	9	8 (9)	8 (5) 9 (4)
<i>B. pseudocatenulatum</i> from infant feces	65	4 (65)	1 (40) 3 (25)
from calf feces	35	4 (28) 5 (7)	3 (35)
from sewage	20	4 (20)	1 (20)
<i>B. catenulatum</i> from infant feces	21	5 (21)	6 (15) 8 (6)
from adult feces	6	5 (6)	6 (6)
from sewage	17	5 (17)	6 (17)

^a The isozymes are numbered in the order of their decreasing rate of electrophoretic migration toward the anode (11).

^b Numbers in parentheses are the numbers of strains that were endowed with the corresponding isozyme.

^c The labels of the strains studied are reported elsewhere (11).

FIG. 7. Diagrammatic representation of the electrophoretic forms of transaldolase and 6PGD in the four new species and in *B. catenulatum* and *B. thermophilum* (*B. ruminale*). The isozymes are numbered as in ref. 11. The migration distances (in centimeters) of the two sets of isozymes were observed in separate runs. The distribution of these isozymes among the strains studied is reported in Table 9.

less than 30% DNA relatedness (19). As more strains of bifidobacteria from different sources have been examined, some new groups have emerged that show high (70% or more) intra-group and moderate (40 to 80%) intergroup relatedness. In addition, these distinct clusters of strains have some phenotypic characteristics, including G+C content of DNA, composition of cell wall peptidoglycan, fermentation of certain carbohydrates, and isozyme pattern of transaldolase and 6PGD, that correlate with their homology patterns. We believe that these entities should be recognized as distinct (but closely related) species.

Detection of such closely related species depends heavily on the choice of reference strains (see Staley and Colwell [23]), but these distinct organisms may well have significance in the evolutionary sense of taxonomy. Further studies, such as ribosomal ribonucleic acid-DNA hybridization, should help in the interpretation of these relationships. Discovery of these phenotypic differences among closely related species is indeed fortunate because some other genetically unrelated species in the genus cannot yet be differentiated phenotypically. For example, *B. adolescentis* is presently phenotypically indistinguishable from *B. dentium*, yet these species show less than 30% homology (12). Similar examples are known in *Pseudomonas* (7) and in *Fusobacterium* and *Bacteroides* (3).

The opinion has been expressed (Brenner, ref. 1, p. 305) that "a taxonomic scheme is useful if

it enables a worker to distinguish between organisms." But the ecological significance of genetically distinct entities is probably relevant, and it may not be appreciated until the phenotypic expressions of the DNA differences are discovered. Meanwhile, recognition of distinct entities which can be confused phenotypically has both practical and scientific merit and should be considered whenever research or diagnostic data are interpreted.

Consequently it is here proposed that the four DNA homology groups described above be accorded species rank, as follows.

1. *Bifidobacterium cuniculi* sp. nov. (cu. ni 'cu. li. L. n. *cuniculus* rabbit; L. gen. n. *cuniculi* of the rabbit).

Cell characteristics. Gram-positive, non-motile, nonsporeforming, stout rods, 2 to 4 μm in length, with irregular contours and small central swellings or knobs. Chains of cells, with apparently pedunculate symmetrical sprouts, occur rarely. Extensive branching occurs only in liquid cultures with fermentable sugars like starch or maltose. In Trypticase-phytone-yeast extract-glucose (TPY) liquid medium, the cells are generally shorter and have nearly regular contours. This general morphology is very similar to that of *B. globosum*; however, the short knobs or protuberances, often at the middle of the cells, are characteristic of *B. cuniculi* cells grown in solid TPY medium (see Fig. 1).

Surface colonies (TPY medium). Smooth, convex, cream to white, glistening and soft; entire edges.

Relationships to oxygen and CO₂. Highly anaerobic; CO₂ has no effect upon oxygen sensitivity and anaerobic growth.

Temperature relationships. Optimum, 36 to 38°C; minimum, about 29°C; maximum, 42.5 to 43°C; no growth occurs at 28.5 or 44°C.

pH relationships. Initial optimum pH is 6.7 to 7.1; growth is retarded at 6.4 and 7.6; no growth occurs at 5.0 or 8.5.

Fermentation pattern. Glucose, galactose, arabinose, xylose, sucrose, maltose, melibiose, starch, and dextrin are fermented. Fructose is fermented regularly only in prereduced media. Ribose, mannose, trehalose, cellobiose, lactose, melezitose, raffinose, mannitol, sorbitol, inulin, salicin, and gluconate are not fermented. As with other bifidobacteria, glycerol, rhamnose, and lactate are not fermented. Lactose and raffinose are characteristically not fermented by this species.

End products from glucose fermentation. The molar ratio of lactic to acetic acid from the fermentation of glucose varies between strains: from 1:5 (strain RA93) to 1:20 (strain RA98). L-(+)-lactic acid is produced. Small amounts of

formic acid are produced. Propionic and butyric acids are not produced.

Biochemical reactions. Catalase and pseudocatalase (hemin) are not produced; nitrate is not reduced; indole is not produced; acetylmethylcarbinol is produced; gelatin is not liquefied; skim milk is unchanged; ammonia is not produced from urea or arginine.

G+C content of the DNA. The G+C content of the DNA is 64.1 ± 0.35 mol% (T_m).

DNA relationships. The DNA of this species showed little or no homology (10 to 30%) with that of any other species (or group) of *Bifidobacterium* except the DNA of the type strain of *B. globosum* (RU230 = ATCC 25864), with which homologies ranged from 50 to 67%.

Amino acid composition of the cell wall peptidoglycan. The interpeptide bridge of the murein of the cell wall of the type strain is Orn(Lys)-Ala(Ser)-Ala₂ (O. Kandler, personal communication).

Isozyme pattern. Produces transaldolase isozyme 1 and 6PGD (oxidized nicotinamide adenine dinucleotide phosphate dependent) isozyme 4; this 1-4 pattern is unique among the bifidobacteria.

Source. Feces of adult rabbits.

Type strain. The type strain is RA93 from the Collection of the Istituto di Microbiologia Agraria, Università di Bologna, Italy. A culture of this strain has been deposited in the American Type Culture Collection, Rockville, Md., under the number 27916.

2. *Bifidobacterium choerinum* sp. nov. (choe. ri 'num. M. L. adj. *choerinus* pertaining to a pig).

Cell characteristics. Gram-positive, non-motile, nonsporeforming, spindle- or lemon-shaped cells, 1.5 to 3.5 μm long when freshly isolated. Cells occur singly or in pairs; after subculture in TPY medium, the cells are distinctly longer (6 to 7 μm), with irregular contours; very few cells have branches. Cells are generally more uniform in shape when grown in liquid TPY medium; they are elongated (10 to 12 μm), slightly bent, and with rounded ends. Some cells with enlarged extremities occur in starlike aggregates (see Fig. 2).

Surface colonies (TPY medium). Smooth, convex, cream to white, glistening, and soft; entire edges.

Relationships to oxygen and CO₂. Slightly anaerobic. Effect of CO₂ not detectable.

Temperature relationships. Optimum, 41 to 42.5°C; minimum, about 27°C; maximum, 44.5 to 45.0°C; no growth at 25 or 46°C.

pH relationships. Initial optimum pH is 6.5 to 7.3; growth is retarded at 6.2 or 7.6; no growth at 5.2 or 8.5 (after 2 days).

Fermentation pattern. Ribose, glucose, ga-

lactose, sucrose, maltose, melibiose, lactose, starch, and dextrin are fermented. Fructose is ordinarily fermented only in prereduced media. Arabinose, xylose, mannose, trehalose, cellobiose, melezitose, mannitol, sorbitol, inulin, salicin, and gluconate are not fermented. As with other bifidobacteria, glycerol, rhamnose, and lactate are not fermented.

End products from glucose fermentation. The molar ratio of lactic to acetic acid from the fermentation of glucose ranges from 1:4 (strain SU806) to 1:8 (strain SU882). L-(+)-lactic acid is produced. Small amounts of formic acid are produced. Propionic and butyric acids are not produced.

Biochemical reactions. Catalase and pseudocatalase (hemin) are not produced; nitrate is not reduced; indole is not produced; acetylmethylcarbinol is produced; gelatin is not liquefied; skim milk is acidified and coagulated; ammonia is not produced from urea or arginine.

G+C content of the DNA. The G+C content of the DNA is 66.3 ± 0.15 mol% (T_m).

DNA relationships. The DNA of this species is essentially unrelated to that of any other species or homology group of *Bifidobacterium* (homologies range from 10 to 30%); exceptions are the DNAs of *B. pseudolongum*, *B. globosum*, and *B. asteroides*, for which the homologies range from 25 to 60%.

Amino acid composition of the cell wall peptidoglycan. The interpeptide bridge of the murein of the cell wall of the type strain is Orn(Lys)-Ser-Ala₂ (O. Kandler, personal communication).

Isozyme pattern. Transaldolase isozyme 3 and 6PGD (oxidized nicotinamide adenine dinucleotide phosphate dependent) isozyme 4 are displayed by all strains of the group tested. This 3-4 pattern is unique among the bifidobacteria.

Source. Feces of suckling piglets.

Type strain. The type strain is SU806 from the Collection of the Istituto di Microbiologia Agraria, Università di Bologna, Italy. A culture of this strain has been deposited in the American Type Culture Collection under the number 27686.

3. *Bifidobacterium boum* sp. nov. (bo' um. L. n. bos a cow; L. pl. gen. n. boum of cattle).

Cell characteristics. Gram-positive, non-motile, nonsporeforming cells, 0.6 to 0.7 by 2.0 to 5.0 μ m, very irregular in shape, generally occurring in pairs; the paired cells form wide angles, with protuberances, knobs, or branchings near the junction. Clumps of branched cells are frequent. Under slight anaerobiosis, extensive and complicated branching is frequent on cells with spatula-like extremities (see Fig. 3). The

sediment in liquid TPY cultures is flaky or in mold-like pellets and is dispersed only with difficulty; the supernatant is clear.

Surface colonies (TPY medium). Smooth, convex, cream to white, glistening, and soft; entire edges.

Relationships to oxygen and CO₂. Rather aerotolerant; grows in air + CO₂. CO₂ is not required for growth under anaerobic conditions.

Temperature relationships. Optimum, 39 to 40°C; minimum, about 25°C; maximum, about 45°C; no growth at 20 or 46°C.

pH relationships. Initial optimum pH is 6.5 to 7.0; growth is retarded at 6.0 or 7.4; no growth at 5.0 or 8.0.

Fermentation pattern. Glucose, fructose, maltose, sucrose, melibiose, raffinose, starch, and dextrin are fermented. Arabinose, xylose, ribose, mannose, melezitose, sorbitol, mannitol, gluconate, glycerol, rhamnose, and lactate are not fermented. Lactose is fermented by 25% of the strains and cellobiose is fermented by 7%; galactose is occasionally slowly fermented.

End products from glucose fermentation. The molar ratio of lactic to acetic acid from the fermentation of glucose is about 1:3. L-(+)-lactic acid is produced. Small amounts of formic acid are produced. Propionic and butyric acids are not produced.

Biochemical reactions. Catalase and pseudocatalase (hemin) are not produced; nitrate is not reduced; indole is not produced; acetylmethylcarbinol is produced; gelatin is not liquefied; skim milk is usually unchanged; ammonia is not produced from urea, asparagine, or arginine.

G+C content of the DNA. The G+C content of the DNA is 60.0 ± 0.2 mol% (T_m).

DNA relationships. The DNA of this species is 60 to 70% related to that of *B. thermophilum* (*B. ruminale*) but is essentially unrelated to any other species (or homology group) of *Bifidobacterium*.

Amino acid composition of the cell wall peptidoglycan. The interpeptide bridge of the peptidoglycan of the cell wall of the type strain is Lys-Ser-Glu (O. Kandler, personal communication).

Isozyme pattern. Produces transaldolase isozyme 6. Also produces 6PGD (oxidized nicotinamide adenine dinucleotide phosphate dependent) isozymes 8, 9, and 9a, which are shared by *B. thermophilum* (*B. ruminale*). The 6-8 pattern is shared by some *B. suis* strains.

Source. Rumen of cattle and feces of pigs; also found in sewage.

Type strain. The type strain is RU917 from the Collection of the Istituto di Microbiologia Agraria, Università di Bologna, Bologna, Italy.

A culture of this strain has been deposited in the American Type Culture Collection under the number 27917.

4. *Bifidobacterium pseudocatenulatum* sp. nov. (pseu. do. ca. te. nu. la' tum. Gr. adj. *pseudēs* false; L. adj. *catenulatum* specific epithet; M. L. adj. *pseudocatenulatum* the false [*B.*] *catenulatum*).

Cell characteristics. Gram-positive, non-

motile, nonsporeforming, irregularly shaped rods, 0.6 to 0.7 by 2 to 5 μ m, generally arranged in pairs which form angles with protuberances, knobs, or short branches near the point of contact. Strains with much more variable and irregular shape are occasionally found, including bifid forms similar to those of *B. bifidum* or pairs of cells with swollen ends similar to those of *B. longum*. Some strains show distorted chains of

TABLE 10. Fermentation patterns of *B. pseudocatenulatum* and *B. catenulatum*^a strains isolated from infant and calf feces and type strains^b

Compound	<i>B. pseudocatenulatum</i>			<i>B. catenulatum</i>	
	From infant feces 100 strains	From calf feces 74 strains	Type strain ATCC 27919	From infant feces 37 strains	Type strain ATCC 27539
L-Arabinose	+ ^c (97/100) ^d	+ (74/74)	+	+ (36/37) ^d	+
D-Xylose	+ (98/100) ^e	+ (74/74)	+	+ (32/37) ^e	+
D-Mannose	+ (68/100) ^f	+ (53/74) ^f	+	- (35/37) ^f	-
D-Cellobiose	+ (71/100) ^g	- (58/74) ^g	+	+ (37/37)	+
D-Trehalose	+ (76/100) ^f	- (41/74) ^f	+	+ (21/37) ^f	-
Mannitol	- (94/100) ^h	- (73/74) ^h	-	- (31/37) ^h	-
Sorbitol	+ (100/100)	+ (39/74) ⁱ	+	+ (37/37)	+
Salicin	+ (87/100) ^f	+ (74/74)	+	+ (30/37) ^f	+
Inulin	- (73/100) ^f	- (69/74) ^f	-	- (30/37) ^f	-
Starch	+ (100/100)	+ (74/74)	+	- (37/37)	-
Glucuronate	-(56/100) ^j	- (74/74)	+	- (24/37) ^j	+

^a The fermentation patterns of *B. catenulatum* strains from sewage and from feces of adult men were reported elsewhere (12). The pattern of the type strain B669 (=ATCC 27539) is reported here in its corrected version (see text).

^b All strains fermented ribose, glucose, galactose, sucrose, maltose, lactose, melibiose, and raffinose. None fermented melezitose, rhamnose, glycerol, or lactate. Several strains of both species showed marginal or no fermentation of fructose (see footnote to Table 8).

^c +, Fermented; -, not fermented.

^d *B. pseudocatenulatum*: strains B2262, B2266, and B2351 were negative. *B. catenulatum*: strain B2124 was negative.

^e *B. pseudocatenulatum*: strains B2263 and B2266 were negative. *B. catenulatum*: strains B2122, B2124, B2238, B2244, and B2245 were negative. The type strain, B669, occasionally fermented arabinose and xylose slowly (12).

^f The behavior toward mannose, trehalose, salicin, and inulin is generally variable in most bifidobacteria; therefore the list of strains was omitted.

^g *B. pseudocatenulatum*. From infants: strains B1280, B1286, B1297, B1330, B1830, B1831, B1832, B1833, B1834, B1886, B1888, B1965, B2067, B2247, B2248, B2249, B2250, B2251, B2252, B2253, B2262, B2263, B2266, B2340, B2351, B2362, B2363, B2366, and B2367 were negative. From calves: VT6, VT74, VT75, VT77, VT354, VT393, VT394, VT396, VT397, VT398, VT399, VT401, VT404, VT405, VT406, and VT409 were positive.

^h *B. pseudocatenulatum*: From infants: strains B1828, B1830, B1831, B1832, B1833, and B1834 (all from the same specimen) were positive. From calves: strain VT9 was positive. *B. catenulatum*: strains B2238, B2241, B2242, B2243, B2244, and B2245 (all from the same specimen) were positive.

ⁱ *B. pseudocatenulatum*. From calves: strains VT6, VT9, VT74, VT75, VT184, VT201, VT202, VT203, VT204, VT205, VT206, VT207, VT254, VT262, VT263, VT265, VT269, VT270, VT337, VT339, VT341, VT348, VT351, VT353, VT354, VT356, VT360, VT368, VT369, VT370, VT371, VT372, VT373, VT374, and VT375 were negative.

^j *B. pseudocatenulatum*: strains B1279, B1280, B1286, B1291, B1297, B1342, B1555, B1557, B1561, B1735, B1736, B1874, B1875, B1879, B1880, B1889, B1892, B1893, B1957, B1960, B1961, B1965, B1966, B1969, B1970, B1973, B1977, B2072, B2073, B2075, B2077, B2078, B2079, B2080, B2082, B2083, B2282, B2283, B2284, B2286, B2287, B2288, B2339, and B2445 were positive. *B. catenulatum*: strains B1331, B1333, B1334, B1338, B1340, B1341, B1738, B1741, B1742, B1744, B1745, B1746, and B1748 were positive.

small coccoid and irregular cells or long, branched, hyphal-like cells (see Fig. 5).

Surface colonies (TPY medium). Smooth, convex, cream to white, glistening, and soft; entire edges.

Relationships to oxygen and CO₂. Anaerobic. CO₂ is without effect upon oxygen sensitivity or anaerobic growth.

Temperature relationships. Optimum, 39 to 41°C; minimum, 28 to 29°C; maximum, 42 to 43°C; no growth at 26 or 44°C.

pH relationships. Initial optimum pH is 6.5 to 6.9; growth is retarded at 6.0 or 7.2; no growth at 4.5 or 8.0.

Fermentation pattern. Ribose, glucose, galactose, sucrose, maltose, lactose, starch, dextrin, melibiose, and raffinose are fermented. Melezitose, rhamnose, glycerol, and lactate are never fermented. Arabinose and xylose are fermented by most strains. Sorbitol is fermented by the strains isolated from feces of infants and by 50% of the isolates from calf feces. Gluconate is fermented by 45% of the strains from infants but not by those from calves. This pattern of fermented carbohydrates is shared by *B. catenulatum* which, however, does not ferment starch or dextrin (Table 10).

End products from glucose fermentation. The molar ratio of lactic to acetic acid from the fermentation of glucose is 1:2. L-(+)-lactic acid is produced. Large quantities of formic acid are produced. Propionic and butyric acids are not produced. CO₂ is produced only in the fermentation of gluconate.

Biochemical reactions. Catalase and pseudocatalase (hemin) are not produced; nitrate is not reduced; indole is not produced; acetylmethylcarbinol is produced; gelatin is not liquefied; skim milk is acidified and coagulated; ammonia is not produced from urea, asparagine, or arginine.

G+C content of the DNA. The G+C content of the DNA is 57.5 ± 0.3 mol% (T_m).

DNA relationships. The DNA of this species is 60 to 80% related to that of *B. catenulatum*; it is essentially unrelated to that of any other species (or homology group) of *Bifidobacterium*.

Amino acid composition of the cell wall peptidoglycan. The interpeptide bridge of the peptidoglycan of the cell wall of the type strain is Lys-Ala₂-Ser (O. Kandler, personal communication).

Isozyme pattern. Produces transaldolase isozyme 4; a few strains from calf feces produce isozyme 5; 6PGD (oxidized nicotinamide adenine dinucleotide phosphate dependent) isozymes 1 and 3 are produced; both 4-1 and 4-3 patterns are unique among the bifidobacteria and differ-

entiate *B. pseudocatenulatum* from *B. catenulatum*, which has the 5-6 pattern.

Source. Abundant in sewage, in the feces of breast- and bottle-fed infants, and in the feces of suckling calves; found also in the feces of adult humans.

Type strain. The type strain is B1279 from the Collection of the Istituto di Microbiologia Agraria, Università di Bologna, Bologna, Italy. A culture of this strain has been deposited in the American Type Culture Collection under the number 27919.

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REPRINT REQUESTS

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